

[19] 中华人民共和国国家知识产权局

[51] Int. Cl.<sup>7</sup>

C07K 14/32

C07K 17/10

## [12] 发明专利申请公开说明书

[21] 申请号 00115346.3

[43] 公开日 2001 年 10 月 10 日

[11] 公开号 CN 1316436A

[22] 申请日 2000.4.6 [21] 申请号 00115346.3

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权利要求书 1 页 说明书 7 页 附图页数 8 页

[54] 发明名称 水溶性缓释重组蛋白质的制备方法

[57] 摘要

本发明提供了一种水溶性缓释重组蛋白质的制备和纯化方法。提供了三种能使蛋白质在生理条件下稳定的水溶性多聚物。通过本发明中的制备方法,我们从混合物中获得了一种单一的新的具有生物学活性的蛋白质。这一单一产品较以前的基因工程蛋白质有明显的优点。它的体内半衰期延长,体内生物活性增强,稳定性增加,免疫原性降低。提供了用相关方法修饰的人生长激素(hGH)、粒细胞集落刺激因子(G-CSF)、白细胞介素-11(IL-11)、和干扰素(IFN)。

ISSN 1008-4274

1. 水溶性缓释重组蛋白质的制备方法包括：
  - (1) 在合适的 pH 条件下，将水溶性多聚物与重组蛋白质进行反应。
  - (2) 获得水溶性多聚物加成至重组蛋白质的氨基酸残基上的混合物。
  - (3) 通过分子筛和离子交换层析分离未反应物及其它副产物，得到单一的水溶性缓释重组蛋白质。
2. 权利要求 1 的制品中所说的水溶性多聚物是指葡聚糖、多聚（n-乙烯吡咯烷酮）、聚乙二醇、丙二醇（polypropylene glycol）均聚体、聚氧化丙烯/氧化乙烯（polypropylene oxide/ ethylene oxide）共聚体、聚氧乙烯多元醇（polyoxyethylated polyols）及聚乙烯醇的化学物质进行化学修饰。
3. 权利要求 1 中所说的重组蛋白质是指白细胞介素-11（IL-11）、人生长激素（hGH）、人粒细胞集落刺激因子（hG-CSF）、和干扰素（IFN）。
4. 权利要求 1 中所说的水溶性缓释重组蛋白质为单一制品。该制品可制成不同的制剂，这些制剂包括药用上可接受的稀释剂、载体或佐剂。
5. 权利要求 1 中的方法，其中所说的水溶性多聚物是指聚氧乙烯（POE）。包括环氧丙基甲基聚氧乙烯（EPO-MPOE），氯丙基甲基聚氧乙烯（CM-POE）及琥珀酰亚胺丙酸酯基甲基聚氧乙烯（SPA-MPOE）。
6. 权利要求 1 中的方法，其中所说的水溶性缓释重组蛋白质是指水溶性缓释白细胞介素-11（POE-IL-11）、水溶性缓释人生长激素（POE- hGH）、水溶性缓释集落细胞刺激因子（POE- G-CSF）、和水溶性缓释干扰素（POE-IFN）。
7. 权利要求 5 中的聚氧乙烯（POE）具有从 4KDa-80KDa 的分子量。
8. 权利要求 5 中所说的水溶性多聚物是符合 GMP 生产要求的，是药用上可以接受的。

## 水溶性缓释重组蛋白质的制备方法

本发明涉及蛋白质修饰领域，具体的说就是蛋白质的氨基酸残基与水溶性多聚物共价修饰的领域。本发明涉及这一具有生物活性的多肽的衍生物。这一化合物在体内生理环境下，能在较长的一段时间内持续的释放出活性多肽。

随着重组 DNA 技术的飞速发展，具有生物学活性的重组活性蛋白质也应运而生。通过基因工程方法可获得蛋白质。为了得到有活性的蛋白质，除了需复性外，还需要进行必要的修饰，如氨基端的酰氨化、豆蔻酰化等。但是，这些蛋白质进入体内后，仍然可能被胃酸、蛋白酶等所降解而失活。

在近二十年的临床应用过程中，逐渐暴露出目前上市的基因工程药物的一些共同缺点。如生物半衰期短，需频繁用药；有抗原性，长期使用产生抗体；保存条件苛刻，较不稳定等。

如何保护蛋白质，阻止其降解，延长其在体内的半衰期，并降低其抗原性，已经成为药物学研究的首要任务。这就需要对蛋白质进行修饰（Francis Focus on Growth Factors, 3:4-10; 1992 年 5 月，由 Mediscript, Morntview Court, Friern Barnet Land, London N20, OLD, UK 出版）

将蛋白质聚氧乙烯大分子化，则能够有效的解决这些问题。目前还没有很好的方法制备并分离出单一的聚氧乙烯大分子化的蛋白质。而且有关此类蛋白质的活性和稳定性也缺乏深入的研究。本发明正是基于这一需要，对以上这些方面作出了具体的阐述。

通过本发明中的制备方法，我们从混合物中获得了一种单一的新的具有生物学活性的蛋白质。这一单一产品较以前的基因工程蛋白质有明显的优点。它的体内半衰期延长，体内生物活性增强，稳定性增加，免疫原性降低。

本发明涉及活性的水溶性缓释重组蛋白质的单一制品及其制备和分离纯化的方法。通过将重组蛋白质与聚氧乙烯进行反应，如 G-CSF 和聚氧乙烯 20,000 反应，经过离子交换和分子筛层析分离纯化，得到单一的聚氧乙烯大分子化的 G-CSF。令人高兴的是，把所得的各个单一样品进行生物活性测定，其中一种聚氧乙烯大分子化的 G-CSF 具有明显的诱导嗜中性粒细胞的快速增殖作用。而且，更具优势的是这种聚氧乙烯大分子化的 G-CSF 的体内生物活性比对照的 G-CSF 更加高，稳定性更好，其在体内的代谢清除率更低。这可能是由于 POE 与 G-CSF 连接使分子变大，或者蛋白质代谢与排泄所要求的细胞受体的相互作用受到空间位阻。同时，聚氧乙烯本身具有良好的生物相容性。

因此，本发明的内容对获得水溶性的稳定的重组蛋白质具有积极的意

义。

本发明涉及活性的水溶性缓释重组蛋白质的单一制品及其制备和分离纯化的方法。以下对此进行详细描述：

首先，本发明涉及水溶性缓释重组蛋白质的制备和分离纯化的方法。

本方法可以将聚氧乙烯大分子化蛋白质。通过选择合适的缓冲液和 pH 值，使活化的聚氧乙烯反应到蛋白质的氨基酸残基上。反应效率达到 70%。在用离子交换和分子筛层析柱，分离产物中的各组份，得到了单一的制品。并通过肽图谱得以证实。

其次，本发明涉及获得的活性水溶性缓释重组蛋白质。

将分离纯化的单一样品进行生物活性的测定和稳定性实验。比较后，确定了一种既具有生物学活性，又水溶液稳定的单一制品。

实例一

#### 1、 重组人 G-CSF 的制备

人 G-CSF 的氨基酸序列如下：

Met Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys  
Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln  
Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val  
Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro  
Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser Gly  
Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu  
Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala Asp Phe Ala  
Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu  
Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg  
Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu Val  
Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro

我们将含有该序列的质粒，转化大肠杆菌进行表达，对获得的菌体蛋白进行复性，以及离子交换，分子筛柱纯化，获得 G-CSF 半成品即可作为聚氧乙烯化的原料。

#### 2、 聚氧乙烯大分子化的 G-CSF 的制备

将 1 中制备的 1.5mg/ml G-CSF 溶液，用 100mM (pH 8.0) 的磷酸钠缓冲液透析过夜，加入溶解于此缓冲液的平均分子量为 20000 的甲氧基环氧丙基聚氧乙烯 (EPO-MPOE) (摩尔比为聚氧乙烯 : G-CSF = 20 : 1)，4°C 缓慢搅拌 16 小时。

#### 3、 产物混合物的分离纯化

将 2 中制备的产物用 20 mM 乙酸钠 (pH 4.0) 透析过夜。然后上样

于 Pharmacia CM SepharoseFF 柱 (1ml 树脂结合 1mg 蛋白质), 以缓冲液 A(20mM 醋酸钠, pH 4.0) 平衡柱。将蛋白质上样, 再以缓冲液 B(1M 的氯化钠), 其浓度梯度为 0-30% 进行浓度梯度洗脱。流速为 3ml/min, 洗脱液在 280nm 处监测, 部分收集蛋白含量大于 0.5mg/ml 的部分。合并不同峰的馏分进行分析 (结果见图 1)。如图所示, 产物 POE-GCSF 通过离子交换层析柱的得率为 70% 左右。

将离子交换层析柱获得的蛋白质进行分子筛层析。柱为 Pharmacia Sephacryl S-200 HR, 300ml。以 20mm 醋酸钠 (pH 4.0) 缓冲液平衡。上样蛋白以 6ml/min 流速洗脱 200min, 在 280nm 处监测蛋白的流出情况, 合并目的蛋白峰 (结果见图 2)。如图所示, 产物 POE-GCSF 通过分子筛层析柱的得率为 95% 以上。

#### 4、产物生物活性测定

##### (1) 体外活性测定

利用对 G-CSF 依赖的细胞株 NFS-60, 在含有 10% 经热灭活的胎牛血清和 G-CSF 的 IMDM 培养基中, 于 37°C, 5% CO<sub>2</sub> 培养 72 小时后。用无 G-CSF 的培养基清洗细胞 2 次, 以每孔 10000 个细胞/ 50μl 加入 96 孔板, 并加入用 IMDM-FBS 配成的 20、40、80、160、320 pg/ml 的美国 Amgen 公司 G-CSF 标准品和自制 POE-GCSF 样品。37°C, 5% CO<sub>2</sub> 培养 48 小时后, 按 CellTiter 96 Aqueous 细胞生长的非同位素检测试剂盒中的说明, 加入新鲜配制的以 20:1 混合的 MTS/PMS 溶液 20 μl /孔, 继续培养 4 小时, 用 BioTek 酶标仪读取 490nm 的值。酶标仪上自带的软件可显示出标准曲线及计算出待测样品的生物活性 (结果见图 3)。如图所示, 采用本发明独特的方法制备得到的 POE-GCSF 样品具有明显的体外诱导嗜中性粒细胞的快速增殖作用。

##### (2) 体内活性测定

选择 ICR 小鼠, 以 10 μg 蛋白质 /kg 剂量静脉注射自制 POE-GCSF 样品, 并以美国 Amgen 公司 G-CSF 标准品作为阳性对照。注射后, 间隔一定时间, 取外周血, 对嗜中性粒细胞进行计数 (结果见图 4)。如图所示, 采用本发明的方法制备得到的 POE-GCSF 样品在体内仍然具有生物学活性。

#### 5、产物等电点测定

分别取样品 POE-GCSF 原液和美国 Amgen 公司 G-CSF 标准品作为对照液及等电点标准液各 2 μl 分别加入 IEF 胶的加样孔中, 进行聚焦。100 V 15 分钟, 200V 15 分钟, 450V 60 分钟, 停止聚焦 (此时电流趋于零)。然后, 胶进行固定和脱色 (结果见图 5)。结果显示, 样品 POE-

GCSF 与 G-CSF 标准品具有相同的等电点。

## 6、产物分子量及胰肽酶切分离图谱分析

### (1) SDS-PAGE 电泳

利用 10% 的变性 SDS 聚丙烯酰胺凝胶电泳, 并用考马斯亮蓝染色 (结果见图 6)。

### (2) 分子排阻 HPLC

采用 TSK gel SW3000 凝胶柱, 以 10mM 磷酸钠缓冲液 (pH7.4) 为流动相, 进行分子排阻 HPLC, 流速为 1.0ml/min, 洗脱液在 280nm 处监测(结果见图 7)。图中结果通过计算机对峰面积进行积分处理后显示, 样品经分子筛层析柱纯化, 纯度达到 98%以上。

### (3) 胰肽酶切分离图谱

500  $\mu$ g POE-GCSF 及对照品 G-CSF 样品真空干燥, 将其溶解在含有 6M 的盐酸胍及 1mM EDTA 的 0.3 M Tris-HCl 中 (pH 8.4)。配成 1mg/950 $\mu$ l 浓度. 样品中加碘乙酸进行 S-羧甲基化, 并在 37  $^{\circ}$ C 反应 20 分钟。然后样品用 Sephadex G-25 快速离心蛋白分离柱(Quick Spin Protein Columns)脱盐处理。加入上述缓冲液至蛋白终浓度为 0.5mg/ml。

将以上蛋白质样品及对照品用蛋白质内切酶 SV8 (酶与底物比为 1:25)消化, 以不加样品的胰酶溶液作空白。25  $^{\circ}$ C 消化 26 小时。取出, 装 Vydac C4 柱, 以溶液 B (95% 乙腈, 5% 水, 0.1% 三氟乙酸) 3% — 76%梯度洗脱, 通过 HPLC 作肽图. 从而说明 POE-GCSF 为单个 POE 化学修饰的产物。

## 7、产物稳定性研究

将 POE-GCSF 的注射液置于 37 $^{\circ}$ C 放置 48 天, 分别于 0 天, 第 6 天, 第 12 天, 第 24 天, 第 36 天, 第 48 天取样, 进行还原型 SDS-PAGE 电泳 (结果见图 8), 及分子排阻 HPLC。结果显示, POE-GCSF 经过 37 $^{\circ}$ C 放置 48 天后, 仍然没有降解, 性质非常稳定。

### 实例二

将 HPLC 纯的重组人生长激素 (hGH) 1.5mg/ml 溶液, 其中人生长激素的氨基酸序列如下:

Met Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys Glu Gln Lys Tyr  
Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile Pro  
Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys Ser Asn Leu Glu Leu  
Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp Leu Glu Pro Val Gln Phe  
Leu Arg Ser Val Phe Ala Asn Ser Leu Val Tyr Gly Ala Ser Asp Ser Asn  
Val Tyr Asp Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln Thr Leu Met Gly

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Arg Leu Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile Phe Lys Gln Thr Tyr  
Ser Lys phe Asp Thr Asn Ser his Asn Asp Asp Ala Leu Leu Lys Asn Tyr  
Gly Leu Leu Tyr Cys Phe Arg Lys Asp Met Asp Lys Val Glu Thr Phe  
Leu Arg Ile Val Gln Cys Arg Ser Val Glu Gly Ser Cys Gly Phe

用 100mM (pH 8.3) 碳酸铵缓冲液透析过夜，加入溶解于此缓冲液的平均分子量为 20000 的甲氧基环氧丙基聚氧乙烯 (EPO-MPOE) (摩尔比为聚氧乙烯 : hGH = 20 : 1)，4°C 缓慢搅拌 16 小时。然后，在 4°C 环境下，以 10mM NaCl / 20mM Tris (pH7.5) 溶液对反应混合物进行超滤 (截流分子量 20kD)。将去除了未反应人生长激素蛋白质的原液上样于 Pharmacia Q Sepharose FF 柱 (1ml 树脂结合 1.2mg 蛋白质)，以缓冲液 A (50mM NaCl / 20mM Tris (pH7.5)) 平衡柱。将蛋白质上样，再以缓冲液 B (1M 的氯化钠)，其浓度梯度为 0-20% 进行浓度梯度洗脱。流速为 3ml/min，洗脱液在 280nm 处监测，部分收集蛋白含量大于 0.5mg/ml 的部分。合并不同峰的馏分进行分析。将离子交换层析柱获得的蛋白质进行分子筛层析。柱为 Pharmacia Sephacryl S-200 HR, 300ml。以 20mm 醋酸钠 (pH 4.0) 缓冲液平衡。上样蛋白以 6ml/min 流速洗脱 200min，在 280nm 处监测蛋白的流出情况，合并目的蛋白峰。通过分离纯化得到单一的 POE-hGH，进行胰肽酶切分离图谱分析。活性测定结果显示其仍然具有促进骨生长、增加骨骼长度的作用。

### 相关专利

|      |           |
|------|-----------|
| 美国专利 | 4,002,531 |
| 美国专利 | 4,179,337 |
| 美国专利 | 4,414,147 |
| 美国专利 | 4,810,643 |
| 美国专利 | 4,894,226 |
| 美国专利 | 4,897,471 |
| 美国专利 | 4,904,584 |
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| 美国专利 | 5,349,052 |
| 美国专利 | 5,773,581 |
| 欧洲专利 | 0098110   |
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| 世界专利 | 9417185   |



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相关文献

Woodland et al., Journal of Medicinal Chemistry, 1973, 16, 897-901; Wise et al.,  
Journal of Pharmacy and Pharmacology, 1978, 30, 686-689  
Proceed Intern. Symp. Control. Rel. Bioact. Mater. 16, (1989) No.268 pp. 509-510;  
Biotechnology 8, (1990) pp. 755-758  
Mumtaz S, Bachhawat BK, Indian J Biochem Biophys 1991 Oct-Dec;28(5-6):346-  
51

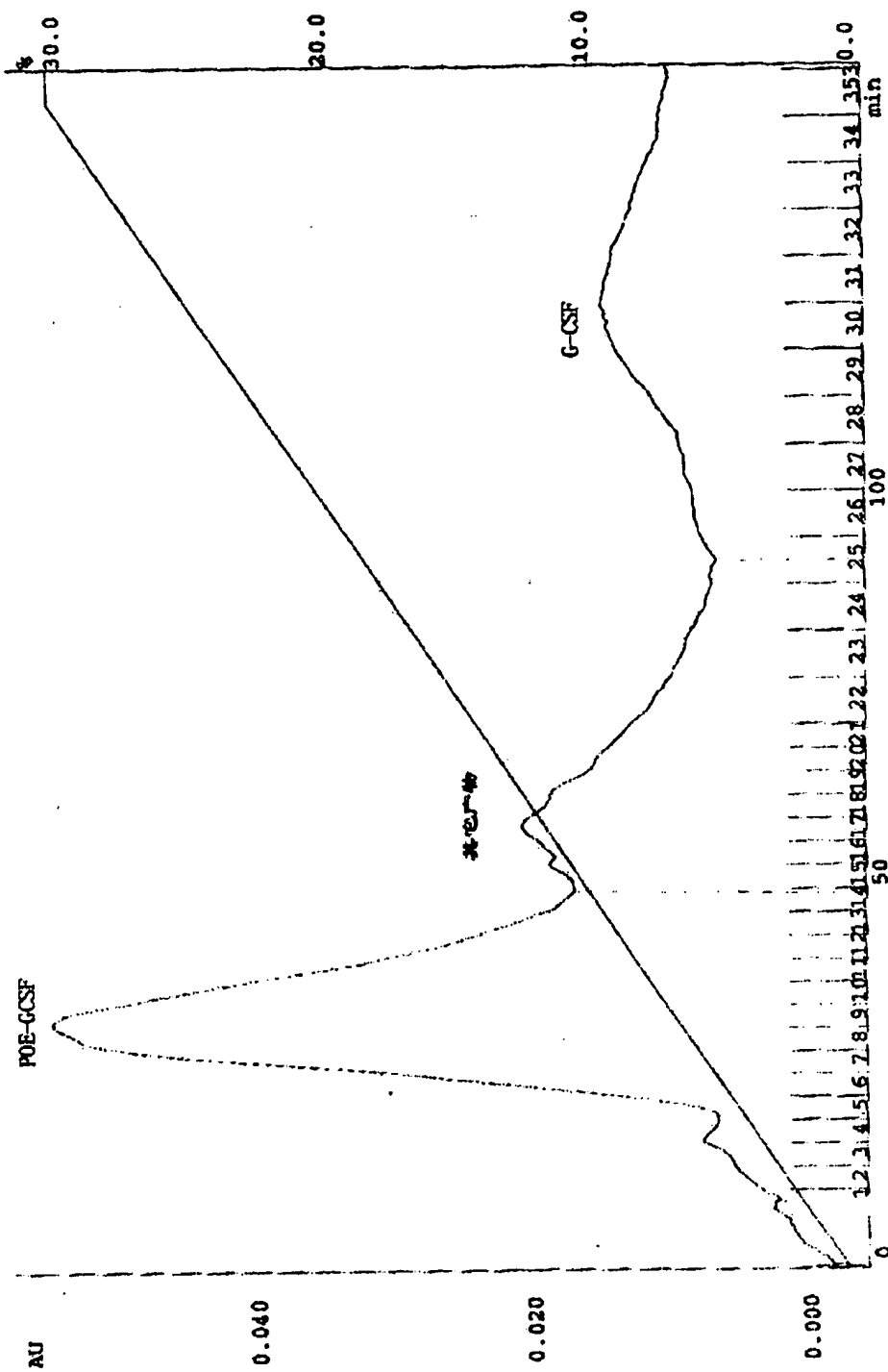


Fig Product thro' IEC column isolation

图1 产物经离子交换层析柱分离

00:04:10

说明书附图

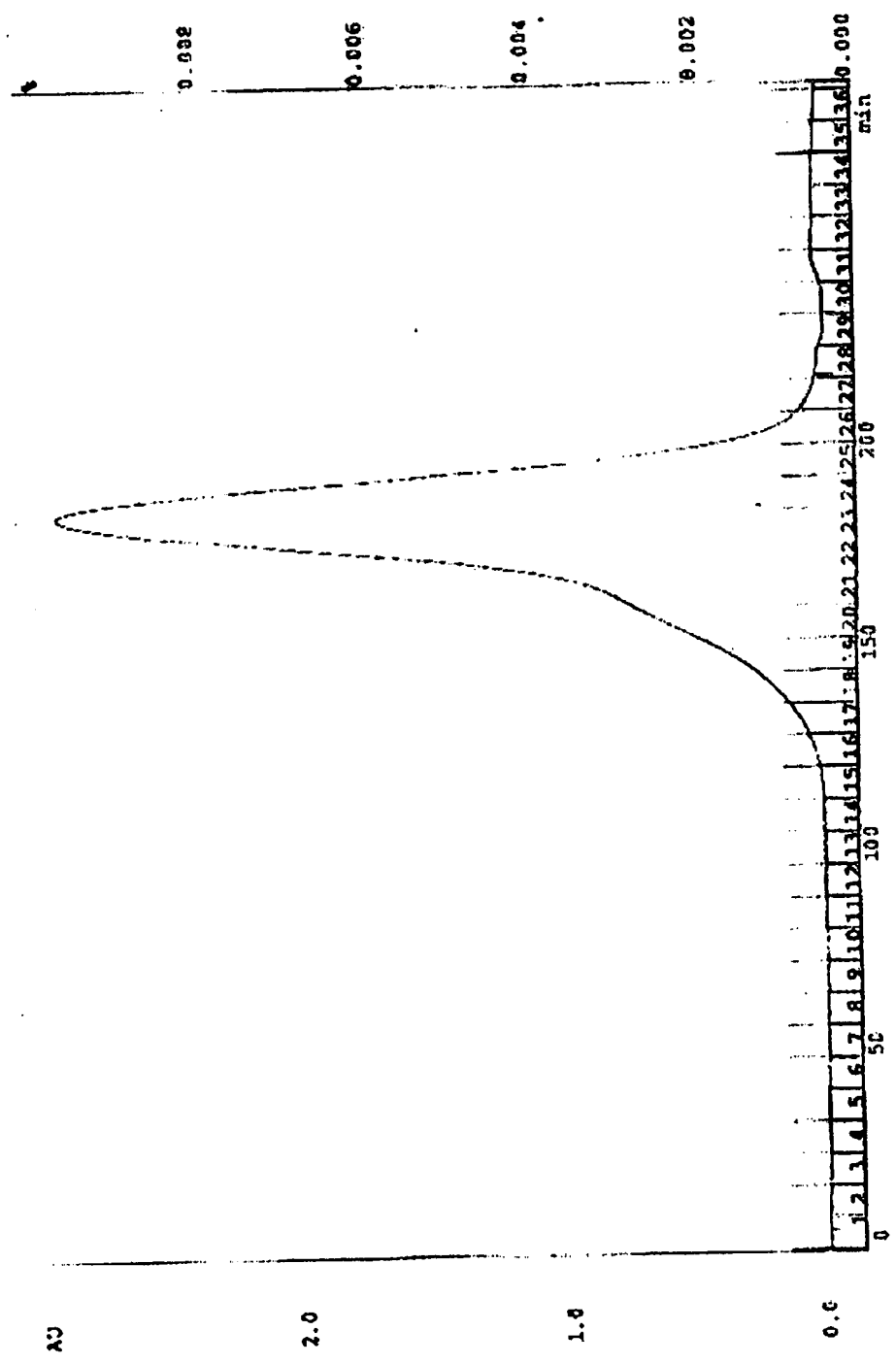


Fig 2 Product thru's MSC column isolation

图2 产物经分子筛层析柱分离

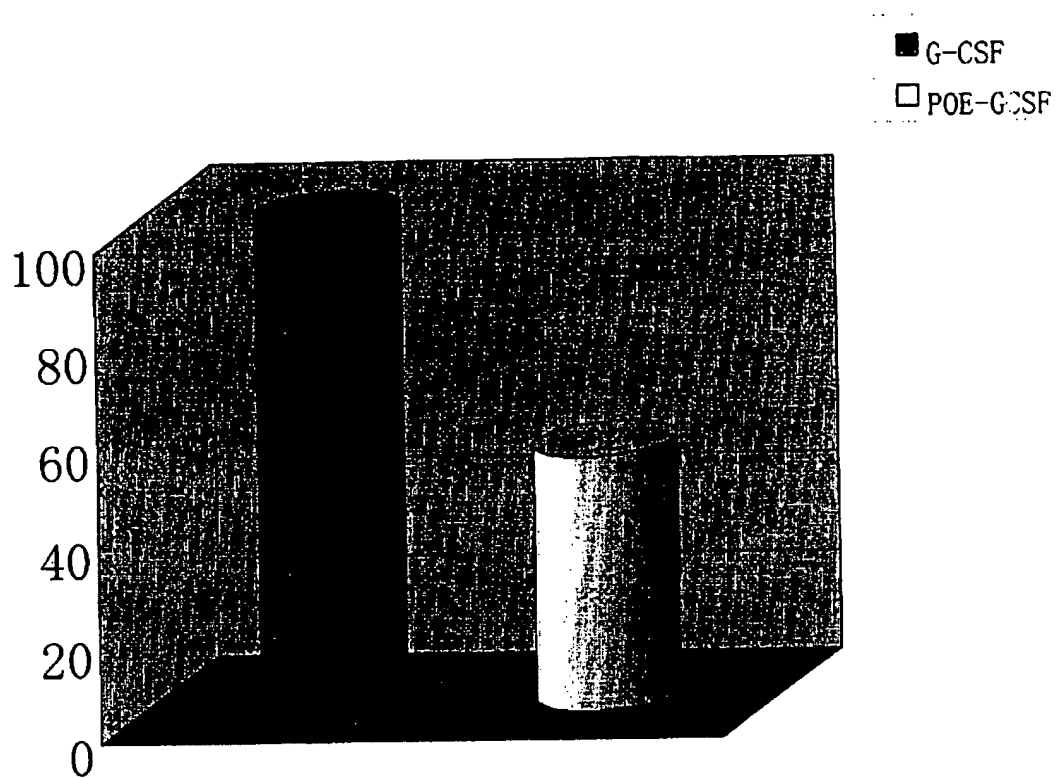


Fig 3. in vitro bioactivity test  
图3 体外生物活性测定

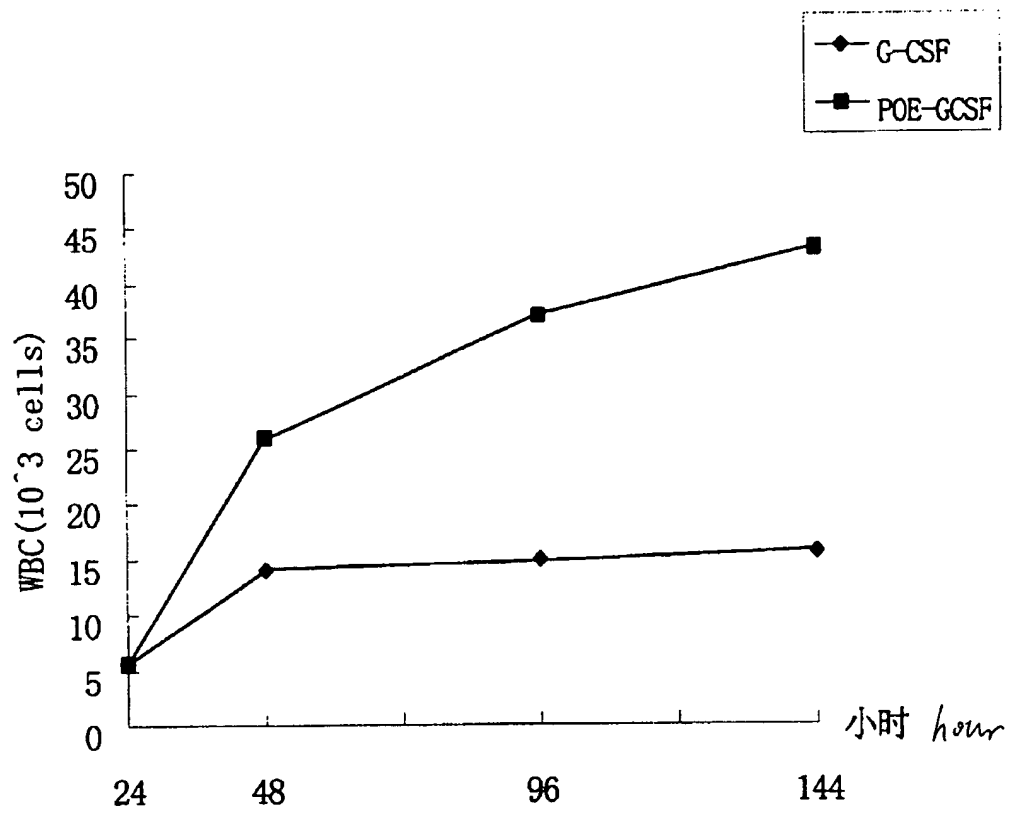
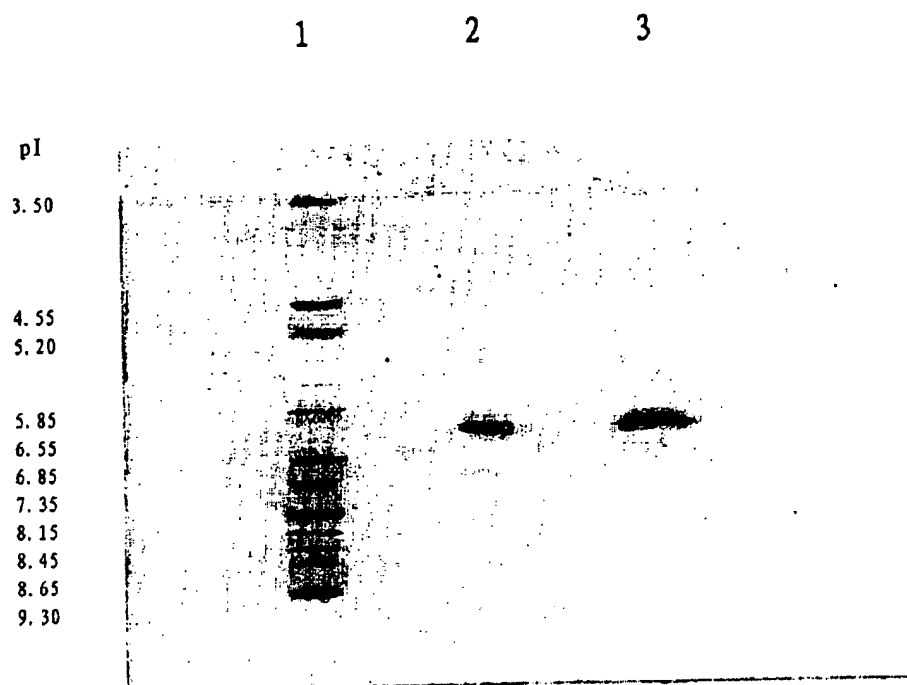
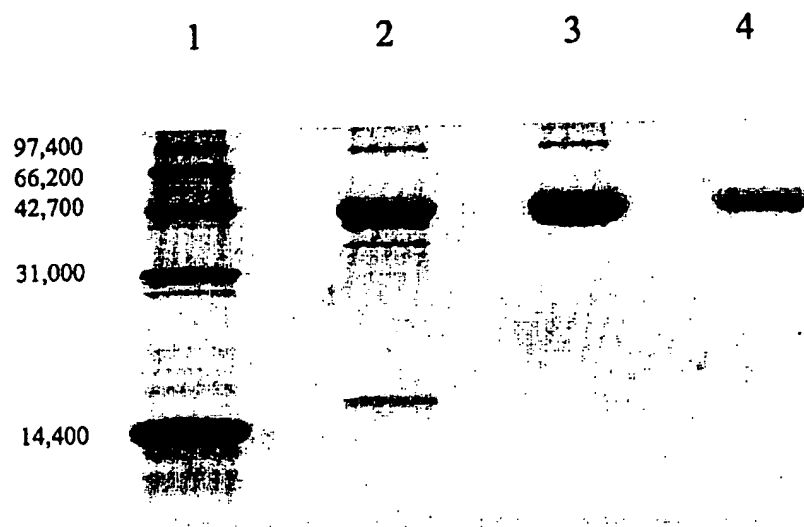


Fig 4. *in vivo* bioactivity test  
图 4 体内生物活性测定



1. 等电点标准品 *isoelectric point standard*
2. GCSF 样品 *sample*
3. POE-GCSF 样品 *sample*

Fig 5. Isoelectric Focusing Electrophoresis Pattern  
图 5 等电聚焦电泳图谱

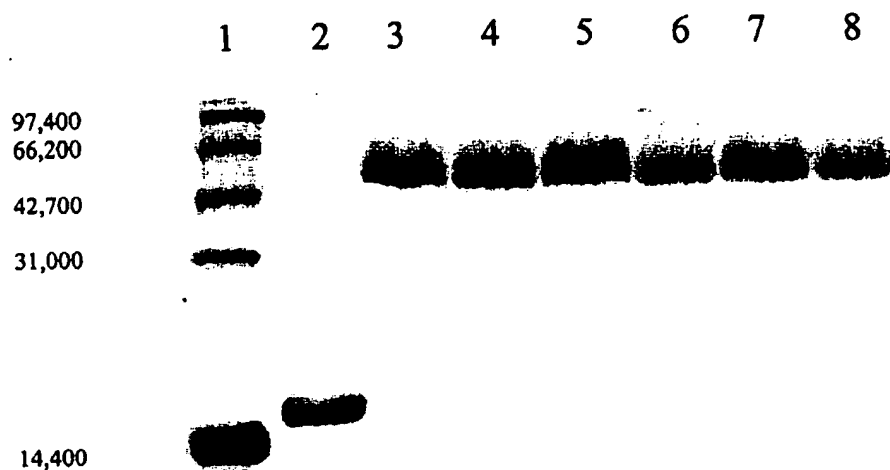


1. 标准蛋白质分子量 *Standard protein MW*
2. 反应后混合物上样液 *reaction mixture*
3. 离子交换柱分离产物 *IEC column isolated product*
4. 分子筛柱分离产物 *MS column isolated product*

*Product and the mixture*  
图 6 产物及反应混合物 SDS-PAGE

00:14:10

说明书附图



1. 标准蛋白质分子量 → Std Protein MW
2. GCSF 样品 → Sample
- 1 st day 3. 第 0 天 POE-GCSF 样品 → Sample
- 6 th day 4. 第 6 天 POE-GCSF 样品
- 12 } day 5. 第 12 天 POE-GCSF 样品
- 24 } 6. 第 24 天 POE-GCSF 样品
- 36 } 7. 第 36 天 POE-GCSF 样品
- 48 } 8. 第 48 天 POE-GCSF 样品

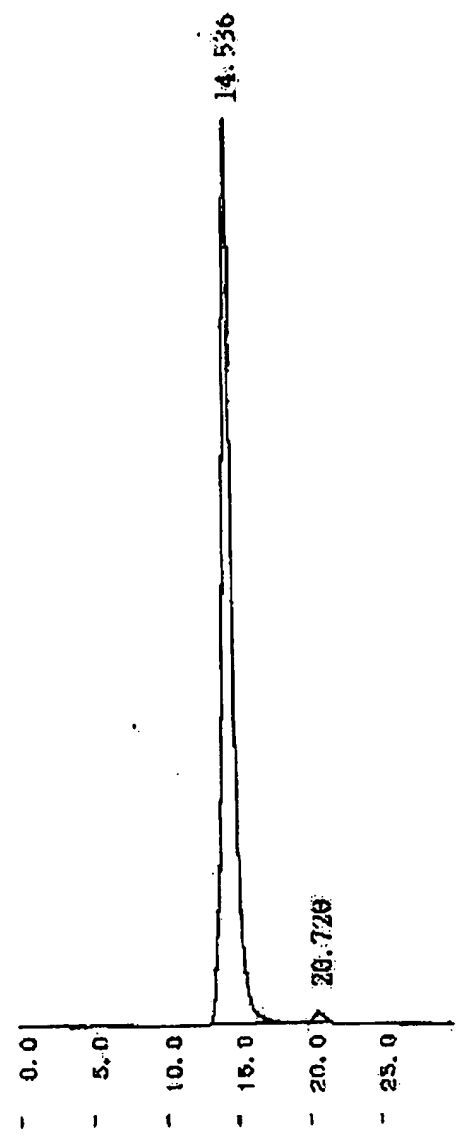
Fig Sample stability  
图 8 POE-GCSF 样品稳定性 SDS-PAGE



00.04.10

说明书附图

C-B7A CHROMATOGRAPH C11=1 HEIGHT No.=4 DATA=P:\SCHRM1.003 00/03/03 14:33:12



| ** CALCULATION REPORT ** |        |        |        |    |      |      |
|--------------------------|--------|--------|--------|----|------|------|
| C11 PRNO                 | TIME   | AREA   | HEIGHT | MR | PDNO | CUNC |
| 1                        | 14.536 | 746833 | 14731  |    |      | 98.5 |
| 2                        | 20.72  | 11430  | 181    | V  |      | 1.5  |
| TOTAL                    |        | 758263 | 14913  |    |      | 100  |

Fig isolated product and excluded  
图7 分离产物分子排阻 HPLC 图谱-pattern

Appl. No. 00115346.3

Publ. No. CN 1316436 A

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### Preparation of Water-soluble Slow-releasing Recombinant Protein

This invention relates to the modification field of protein, more particularly, the covalent modification field of amino acid residue of protein with water-soluble polymer. This invention relates to the polypeptide derivatives having bioactivity. This compound is capable of sustained releasing active peptide over a longer period of time.

Along with the rapid development of recombinant DNA tech, the recombinant active protein having biological activity emerges as the times require. The protein can be obtained by gene engineering. In order to obtain the protein having activity, necessary modification is required except nature-recovery, such as the amidation and cardacylation of N-end etc. However, while proteins enter into body, they can still be degraded by gastric acid, proteinase and deactivated.

In the course of recent 20 years' clinical practice, some common disadvantages of genetically engineered drug appeared on the market have been exposed gradually, such as short biological half-life, frequent administration, having immunogenicity, antibody production in long term use, harsh terms for preserve unstability etc.

How to protect protein, to prevent its degradation, to prolong its in vivo half-life, and to reduce its immunogenicity, have been the most important task of pharmaceuticals study. The modification of protein is quite necessary (Francis Focus on Growth Factors, 3:4-10; 1992, May, Mediscript, Morntview Court, Friern Barnet Land, London N20, OLD, UK). These problems can be effectively solved by making the protein POE

macromolecularized. At present, there is still no very good method for preparing and isolating the single POE macromolecularized protein, and the activity and stability of this kind of protein are short of study in a deepgoing way. This invention is exactly in view of this needs, to explain the abovementioned aspects.

By way of the preparation of this invention, a new single protein having biological activity has been obtained from the reaction mixture. This single product, compared with the prior genetically engineered protein, has marked advantages: prolonged in vivo half-life, enhanced in vivo bioactivity, increased stability, and reduced immunogenicity.

This invention relates to a single product of an active water-soluble slow-releasing recombinant protein, and a method for its preparation and purification. By way of the reaction of recombinant protein with POE, such as the reaction of G-CSF with POW (20kDa), a single POW macromolecularized G-CSF is obtained through ion-exchange and MS chromatographically isolating & purifying. It is most heartening that the POE macromolecularized G-CSF after bioactivity test, has markedly rapid proliferation of induced neutrophile. And having more advantages in, compared with G-CSF control, the POE macromolecularized G-CSF has higher in vivo bioactivity, better stability and lower metabolic clearance in vivo. This is possibly because of molecular size, or the interaction of cell acceptors suffered steric hindrance for protein metabolism, while POE it self has good biocompatibility. Hence, the content of this invention for water-soluble and stable recombinant protein has a positive significance.

This invention relates to a single product of an active water-soluble slow-releasing recombinant protein and a method for its preparation and purification. It is described in detail as follows:

Firstly, this invention relates to a method for preparing and purifying water-soluble slow-releasing recombinant protein.

This method can macromolecularize the protein with polyoxyethylene. By way of selecting suitable buffer solution and pH, activated polyoxyethylene is bonded to the aminoacid residue of the protein, the reaction efficiency is up to 70%. Using ion exchange and MS chromatographic columns, each component in the product is isolated a single preparation has been obtained, and confirmed by the peptide mapping.

Secondly, this invention relates to an active water-soluble slow-releasing recombinant protein obtained. The results of bioactivity test and stability experiment indicate: this isolated and purified single preparation is both biologically active and stable in aqueous solution.

### Example 1

#### 1. Preparation of Recombinant h G-CSF

The amino acid sequence of h G-CSF is as follows:

Met Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys  
Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln  
Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val  
Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro  
Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser Gly  
Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu  
Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala Asp Phe Ala  
Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu  
Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg  
Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu Val  
Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro

The somatic protein obtained from the E.coli expression plasmid containing said sequence, was nature-recovered, ion-exchanged, and MS chromatographically purified, a G-CSF semiproduct obtained capable of being the raw material for POE ylation.

## 2. Preparation of POE macromolecularized G-CSF

1.5mg/ml G-CSF solution from 1, was dialyzed overnight with 100mM (pH 8.0) of Na-phosphate buffer solution. (containing methyl epoxypropyl (EPO-M) POE, ov.MW 20kDa, mol ratio POE:G-CSF=20:1) while stirring slowly at 4°C for 16hr.

## 3. Isolating and Purifying the Product Mixture

The product from 2 was dialyzed overnight with 20mM Na acetate solution (pH 4.0), and applied to Pharmacia CM Sepharose FF column (1ml resin bonded to 1mg protein), the column balanced with buffer solution A (20mM Na acetate, pH 4.0). The protein was applied, and eluted with buffer solution B (1M NaCl, concn gradient 0~30%, flow rate 3ml/min). The eluent was observed at 280nm, the part of protein content > 0.5mg/ml was collected. The fractions of different peaks were combined and analyzed (result see Fig.1). As shown in Fig. The yield of product POE-G-CSF through ion-exchange chromatographic column is ~ 70%.

The protein obtained from ion-exchange chromatographic column was MS chromatographed, the column was Pharmacia Sephacryl S-200HR, 300ml, balanced with 20mm Na acetate buffer solution (pH 4.0). The applied protein was eluted at flow rate 6ml/min for 200min, observed at 280nm, and combined the object protein peaks (result see Fig.2). As shown in Fig, the yield of product POE-G-CSF though MS chromatographic column is > 95%.

## 4. Biological Activity of Product

### (1) in vitro activity test

The cell strain dependent to G-CSF, in IMDM medium containing heat inactivated 10% fetal bovine serum and G-CSF, after cultured under 5% CO<sub>2</sub> at 37°C for 72hr, was washed twice with G-CSF-free medium. 10k cells/50ul/hole, G-CSF standard sample (20, 40, 80, 160, 320pg/ml prepared from IMDM-FBS, Amgen Co, US) and self-made POE-G-CSF sample were added to a culture plate (96 holes). After cultured under 5% CO<sub>2</sub> at 37°C for

48hr, according to the spec of non-isotopic detection agent box (cell Titer 96 aqu, cell growth), 20ul/hole of the freshly prepared (20:1) MTS/PMS mixed solution was added, further cultured for 4hr, the value at 490nm was fetched with BioTek enzyme labeler. The standard curve indicated and the calculated bioactivity of the sample to be determined by the self-contained software are shown in Fig.3. The result indicates: the POE-G-CSF sample prepared from the unique method of this invention has markedly rapid proliferation of in vitro induced neutrophile.

#### (2) in vivo activity test

ICR mice was given an intravenous injection of self-made POE-G-CSF sample at a dose of 10 $\mu$ g protein/kg, and the G-CSF standard sample (Amgen Co, US) as positive control (result see Fig.4). As shown in the Fig, the POE-G-CSF sample prepared by the method of this invention still has in vivo bioactivity.

#### 5. Isoelectric Point test of the Product

2 $\mu$ l each of POE-G-CSF primary liquid, G-CSF standard as control, and isoelectric point standard were added separately into the sampling holes of IEF gel, and focused at 100V for 15min, 200V for 15min, & 450V for 60min, the focusing was stopped (this moment the current goes into O). Then the gel was fixed and decolorized (shown in Fig.5). The result indicates: POE-G-CSF sample and G-CSF sample have the same isoelectric point.

#### 6. Pattern Analysis of MW and Pancreatopertidase cut Isolation of the Product

##### (1) SDS-PAGE Electrophoresis

10% modified SDS polyacrylamide gel electrophoresed and sapphirine colored (result see Fig.6).

##### (2) Molecular Exclusion HPLC

Using TSK gel SW 3000 gel column, saturated with 10mM Na phosphate buffer solution (pH 7.4) as mobile phase, molecular exclusion

HPLC phed at 1.0ml/min, eluent monitored at 2.80nm (result see Fig.7). The product after MS chromatographically purifying has been up to 98% (purity).

### (3) Pancreatopeptidase cut Isolation Pattern

500 $\mu$ g of POE-G-CSF and G-CSF sample (control) were vacuum dried, and dissolved in 0.3M Tris-HCl solution (p.H 8.4, containing 6M guanidine HCl and 1mM EDTA), made the concentration to 1mg/950 $\mu$ l, the sample was S-Carboxy methylated by adding iodo acetic acid, for 20min at 37°C. Then the sample was desalted using Sephadex G-25 Quick Spin Protein Column. Said buffer solution was added to make the final protein concentration to 0.5mg/ml.

The above protein sample and the control were digested with proteolytic enzyme SV8 (enzyme: substrate 1:25), the sample-free pancreatic solution as the blank, at 25°C for 26hr. The digested mixture was applied to Vydac C<sub>4</sub> column, 3% ~ 76% gradiently eluted with solution B (95% acetonitrile, 55 water, 0.1% trifluoroacetic acid), by way of HPLC peptide map, the POE-G-CSF is a single POE chemically modified product.

### 7. Study of the Product Stability

The POE-G-CSF injection was stood at 37°C for 48days, and sampled separately at 0, 6, 12, 24, 36, 48 days, reductive SDS-PAGE electrophoresed (result see Fig.8) and molecular exclusion HPLC. The result indicates: after standing at 37°C for 48 days, the POE-G-CSF is still undegraded, i.e. very stable.

### Example 2

The aminoacid sequence of Recombinant hGH is as follows:

Met Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys Glu Gln Lys Tyr  
Ser Phe Leu Glu Asn Pro Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile Pro  
Thr Pro Ser Asn Arg Glu Glu Tir Gln Gln Lys Ser Asn Lcu Glu Leu  
Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp Leu Glu Pro Val Gln Phe

Leu Arg Ser Val Phe Ala Asn Ser Leu Val Tyr Gly Ala Ser Asp Ser Asn  
Val Tyr Asp Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln Thr Leu Met Gly  
Arg Leu Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile Phe Lys Gln Thr Tyr  
Ser Lys phe Asp Thr Asn Ser His Asn Asp Asp Ala Leu Leu Lys Asn Tyr  
Gly Leu Leu Tyr Cys Phe Arg Lys Asp Met Asp Lys Val Glu Thr Phe  
Leu Arg Ile Val Gln Cys Arg Ser Val Glu Gly Ser Cys Gly Phe

1.5mg/ml of said HPLC pure recombinant hGH solution was dialyzed overnight with 100mM  $\text{NH}_4$  carbonate buffer solution (pH 8.3), methyl epoxypropyl (EPO-M) POE (mol ratio POE: hGH 20:1) while stirring slowly at 4°C for 16hr. The reaction mixture was ultra filtrated (cut off MW 20kDa) with 10mM NaCl/20mM Tris solution (pH 7.5) at 4°C. The primary liquid of unreacted hGH-free protein was applied to Pharmacia Q Sepharose FF column (1ml resin bound to 1mg protein), the column balanced with buffer solution A (50mM NaCl/20mM Tris, pH 7.5). The protein was applied, and 0% ~ 20% gradiently eluted with buffer solution B (1M NaCl) at flow rate 3ml/min. The eluent was monitored at 280nm, the portion containing protein > 0.5mg/ml was partially collected, the cut of different peaks was combined and analyzed. The protein obtained from ion-exchange chromatographic column was MS chromatographed (Pharmacia Sephacryl S-200HR, 300ml), and balanced with 20mM Na acetate buffer solution (pH 4.0). The applied protein was eluted at 6ml/min for 200min, the protein outflow was monitored at 280nm, the object protein peaks were combined. A single POE-hGH after isolating and purifying, was analyzed for pancreatic-peptidase cut isolation pattern. The result of activity test indicates: the water-soluble slow-releasing recombinant protein still has the effect of accelerating the growth of bone and increasing the length of skeleton.



## Claims

1. A method for preparing water-soluble slow-releasing recombinant protein comprising:

(1) reacting a water-soluble polymer with a recombinant protein at appropriate condition;

(2) obtaining a mixture of water-soluble polymer added to amino acid residue of the protein;

(3) obtaining a single water-soluble slow-releasing recombinant protein by isolating the unreactant and other side product using molecular sieve and ion-exchange chromatography.

2. The product of claim 1, wherein said water-soluble polymers are the chemical modifications of: glucosan, poly (n-vinyl pyrrolidone) polyethylene glycol, polypropylene glycol homopolymer, polypropylene oxide/ethylene oxide copolymer, polyoxyethylated polyols and polyvinyl alcohol.

3. The product of claim 1, wherein said recombinant protein is interleukin-11 (IL-11), human growth hormone (hGH), human granulocyte colony stimulating factor (hG-CSF), and interferon (IFN).

4. The product of claim 1, wherein said water-soluble slow-releasing recombinant protein is a single product capable of manufacturing into various preparations which include pharmaceutically acceptable diluent, carrier or adjuvant.

5. The method of claim 1, wherein said water-soluble polymer is polyoxyethylene (POE), including epoxypropyl methyl POE (EPO-MPOE), chloropropyl methyl POE (CM-POE) and succinimidopropionyloxy methyl

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POE (SPA-MPOE).

6. The method of claim 1, wherein said water-soluble slow-releasing recombinant protein is water-soluble slow-releasing interleukin-12 (POE-IL-11), water-soluble slow-releasing human growth hormone (POE-hGH), water-soluble slow-releasing granulocyte colony stimulating factor (POE-G-CSF), and water-soluble slow-releasing interferon (PEO-IFN).

7. The product of claim 5, wherein said polyoxyethylene (POE) has a molecular weight of 40kDa ~ 80kDa.

8. The product of claim 5, wherein said wherein said water-soluble polymer has met the production requirements of GMP, and is pharmaceutically acceptable.

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### Abstract

This invention provides a method for preparing and purifying water-soluble slow-releasing recombinant protein. Three water-soluble polymers which are capable of stabilizing the protein at physiological conditions are provided. By way of the method of this invention, new single protein having biological activity has been obtained. This single product, compared with the prior genetically engineered protein, has marked advantages: prolonged in vivo half-life, enhanced in vivo bioactivity, increased stability, and reduced immunogenicity. The chemical modifications of human growth hormone (hGH), granulocyte colony stimulating factor (G-CSF), interleukin-11 (IL-11), and interferon (IFN) have been obtained.

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